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ORIGINAL ARTICLE

Marked response to cabazitaxel in prostate cancer xenografts expressing androgen receptor variant 7 and reversion of acquired resistance by anti-androgens

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Abstract

Background: Taxane treatment may be a suitable therapeutic option for patients with castration-resistant prostate cancer and high expression of constitutively active androgen receptor variants (AR-Vs). The aim of the study was to compare the effects of cabazitaxel and androgen deprivation treatments in a prostate tumor xenograft model expressing high levels of constitutively active AR-V7. Furthermore, mechanisms behind acquired cabazitaxel resistance were explored.

Methods: Mice were subcutaneously inoculated with 22Rv1 cells and treated with surgical castration ($n = 7$), abiraterone ($n = 9$), cabazitaxel ($n = 6$), castration plus abiraterone ($n = 8$), castration plus cabazitaxel ($n = 11$), or vehicle and/or sham operation ($n = 23$). Tumor growth was followed for about 2 months or to a volume of approximately 1000 mm³. Two cabazitaxel resistant cell lines; 22Rv1-CabR1 and 22Rv1-CabR2, were established from xenografts relapsing during cabazitaxel treatment. Differential gene expression between the cabazitaxel resistant and control 22Rv1 cells was examined by whole-genome expression array analysis followed by immunoblotting, immunohistochemistry, and functional pathway analysis.

Results: Abiraterone treatment alone or in combination with surgical castration had no major effect on 22Rv1 tumor growth, while cabazitaxel significantly delayed and in some cases totally abolished 22Rv1 tumor growth on its own and in combination with surgical castration. The cabazitaxel resistant cell lines; 22Rv1-CabR1 and 22Rv1-CabR2, both showed upregulation of the ATP-binding cassette sub-family B member 1 (ABCB1) efflux pump. Treatment with ABCB1 inhibitor elacridar completely restored susceptibility to cabazitaxel, while treatment with AR-antagonists bicalutamide and enzalutamide partly restored susceptibility to cabazitaxel in both cell lines. The cholesterol biosynthesis pathway was induced in the 22Rv1-CabR2 cell line, which was confirmed by reduced sensitivity to simvastatin treatment.

Conclusions: Cabazitaxel efficiently inhibits prostate cancer growth despite the high expression of constitutively active AR-V7. Acquired cabazitaxel resistance involving overexpression of efflux transporter ABCB1 can be reverted by bicalutamide or enzalutamide treatment, indicating the great clinical potential for combined treatment with cabazitaxel and anti-androgens.

KEYWORDS

ABCB1, androgen receptor, cabazitaxel, cholesterol, prostate cancer, splice variant

1 | INTRODUCTION

Prostate cancer is one of the most commonly diagnosed malignancies and a leading cause of cancer mortality amongst men living in developed countries.¹ Advanced prostate cancer is treated with androgen deprivation therapy, which is initially efficient in most cases, but eventually gives disease progress into a lethal stage known as castration-resistant prostate cancer (CRPC). The suggested underlying mechanisms behind CRPC include androgen receptor (AR) amplifications, AR mutations, constitutively active AR variants, intracrine steroid synthesis, and AR bypassing mechanisms.² For many years, docetaxel was the only available treatment for CRPC, but now several novel therapies with different mechanisms of action are approved³; abiraterone, a steroidogenesis inhibitor blocking the CYP17A1 enzyme, the novel AR antagonist enzalutamide, the radioisotope radium-223, the immunotherapy Sipuleucel-T, and a new tubulin-blocking taxane, cabazitaxel.⁴⁻⁸

In a previous study, we identified a sub-group of patients with CRPC with bone metastases expressing high levels of the constitutively active AR variant 7 (AR-V7) having a very poor prognosis.⁹ Further research has shown that AR-V7 messenger RNA (mRNA) detection in circulating tumor cells or in peripheral blood of patients with CRPC indicates a likely resistance to treatment with enzalutamide and abiraterone, but not to taxanes, suggesting that cabazitaxel might be a suitable treatment for patients with high tumor expression of AR variants.¹⁰⁻¹⁵

Therefore, the aim of this study was to investigate the effects of cabazitaxel on human 22Rv1 prostate cancer xenografts, expressing high levels of constitutively active AR-V7 along with other AR variants (AR-V1-6, V9, V12-14),¹⁶⁻¹⁹ in comparison to effects of surgical castration and abiraterone treatment. We also wanted to explore mechanisms behind acquired cabazitaxel resistance. For this purpose, cabazitaxel resistant cell lines were established.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The 22Rv1 cell line (American Type Culture Collection [ATCC], CRL-2505) was maintained according to ATCC instructions in RPMI 1640 + GlutaMAX supplemented with 10% fetal bovine serum (FBS), 100 U penicillin/mL and 100 µg streptomycin/mL, 10 mM HEPES and 1 mM sodium pyruvate (Thermo Fisher Scientific). Cabazitaxel resistant

cell lines (below) were maintained in RPMI with 10% charcoal-stripped FBS (Thermo Fisher Scientific).

2.2 | Treatment of 22Rv1 xenografts

The 22Rv1 cells (1.2×10^6) were diluted 1:1 in RPMI (Thermo Fisher Scientific) and Matrigel (BD biosciences) before injected subcutaneously into the flanks of 8-weeks-old, athymic male BALB/c nude mice (Scanbur, Karlslunde, Denmark). Tumor volume was measured two to three times per week by calipers and calculated by $\text{length} \times (\text{width}^2)/2$. When tumors reached approximately 100 to 200 mm³, mice were randomized and selected for treatment with castration by surgical incision ($n = 7$), abiraterone ($n = 9$), cabazitaxel ($n = 6$), castration plus abiraterone ($n = 8$), or castration plus cabazitaxel ($n = 11$) of which six animals received repeated cabazitaxel treatment at tumor regrowth. Control animals received sham operation ($n = 5$), vehicle for abiraterone ($n = 5$) or cabazitaxel ($n = 5$), or sham operation plus vehicle for abiraterone ($n = 2$) or cabazitaxel ($n = 6$). Abiraterone acetate (kindly provided by Janssen Cilag AB) was diluted to 40 mg/mL in 5% benzyl alcohol, 95% safflower oil and given daily by intraperitoneal injections of 0.5 mmol/kg. Cabazitaxel was received as frozen aliquots of Jevtana (Sanofi) 10 mg/mL, 24% polysorbate 80, 9.8% ethyl alcohol (EtOH) stock solution (leftovers from patient treatments at the Oncology clinic, Umeå University Hospital) and diluted to 2.08 mg/mL in 5% polysorbate 80, 5% glucose and 2% EtOH before given as two injections of 20 mg/kg with 7 days in-between. Mice that showed a body weight loss <10% (5 out of 17) received a third injection. The experiment was terminated after approximately 2 months or when tumors reached a volume of about 1000 mm³. Tumors and prostate tissue were dissected, freshly processed or fixed in 4% paraformaldehyde. Animal work was carried out in accordance with the protocol approved by the Umeå Ethical Committee for Animal Studies (permit number A5-15).

2.3 | Establishment of cabazitaxel resistant cell lines

Two different 22Rv1 xenografts relapsing during repeated cabazitaxel treatment were established as cell lines; termed 22Rv1-CabR1 and 22Rv1-CabR2, as further described. Tumor tissue was aseptically minced using scissors and dissolved by 0.1% collagenase (Sigma-Aldrich) in

Hanks' balanced salt solution (HBSS) containing calcium and magnesium (Thermo Fisher Scientific) while incubated at 37°C for 1 hour. After incubation, cells were filtered through a 100 µm cell strainer and washed with HBSS free from calcium and magnesium. Filtered cells were centrifuged twice, resuspended in growth media (described above) and seeded. When the cells showed stable growth the media was changed to RPMI with 10% charcoal-stripped FBS and increasing concentrations of cabazitaxel from 0.5 to 10 nmol/L within five passages. Cells were grown without cabazitaxel for at least one passage before experiments. 22Rv1 cells grown in charcoal-stripped media together with vehicle were used as control.

2.4 | Evaluation of cabazitaxel resistance

Resistance to cabazitaxel was tested in vitro by growing triplicates of 2.5×10^5 cells in six-well plates for 9 days in media containing 0.01, 0.1, 1.0, 10, 20, or 100 nmol/L cabazitaxel before counting viable cells using a Countess automated cell counter (Thermo Fisher Scientific).

To examine if 0.25 µmol/L elacridar (Sigma-Aldrich), 20 µmol/L bicalutamide (Sigma-Aldrich) or 20 µmol/L enzalutamide (Selleckchem) could reverse the in vitro cabazitaxel resistance, the resistant cell lines were grown in quadruplicates of 1×10^4 cells in 96-well plates (IsoplateTC, Wallac, Finland) for 96 hours in media containing 0 to 10 nmol/L cabazitaxel ± each inhibitor. Cell viability was assayed with CellTiter Glo 2.0, according to the manufacturer's instructions (Promega). Luminescence was measured using a SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices).

To confirm cabazitaxel resistance in vivo, xenografts were established from 22Rv1-CabR1 ($n = 10$) and 22Rv1-CabR2 ($n = 7$) cells by subcutaneous injections, as described above. 22Rv1 cells were used as control ($n = 8$). All mice were surgically castrated 4 days before tumor cell injections. Two rounds of cabazitaxel treatment were given (20 mg/kg each, 7 days in-between) when tumors reached the size of 100 to 200 mm³. When tumors reached a volume of approximately 1000 mm³, mice were killed. Tumor tissue was collected and processed as described above.

2.5 | Simvastatin sensitivity

Simvastatin was purchased from Sigma-Aldrich and activated according to the protocol from the vendor. Resistance to simvastatin was tested in vitro by growing quadruplicates of 1×10^4 cells in 96-well plates for 4 days in media containing 0 to 100 µmol/L simvastatin. Cell viability was then assayed using CellTiter Glo as described above.

2.6 | Androgen receptor activity

The Signal Lenti Reporter assay (Qiagen) was used to determine the AR activity of the cells, according to the protocol from the vendor.

Briefly, cells were seeded in 96-well plates (1×10^4 cells/well) and incubated overnight before transduced in triplicate with either AR Luc (cat no: CLS-8019L) or negative control (CLS-NCL) at multiplicity of infection (MOI) of 10 in 50 µL serum-free transducing media. The next morning, 10% charcoal-stripped FBS-HI culture media was added containing 0 or 1 nM dihydrotestosterone (DHT). After 72 hours, AR activity was measured as luciferase activity using the luciferase substrate (LARII) of the Dual-Luciferase Reporter Assay (Promega). Relative AR activities were obtained by dividing signals with the corresponding negative controls.

2.7 | Prostate-specific antigen measurements

Cells were seeded (3×10^5 cells/well in six-well plates) and incubated with or without 1 nM DHT for 4 days. Both cells and conditioned media were harvested. The total number of cells per well was counted using a Countess automated cell counter. Cells and cell debris were removed from the conditioned media (centrifugation 3000g, 5 minutes) before analysis of total prostate-specific antigen (PSA) levels, according to the clinical routine at the accredited Umeå University hospital laboratory (Elecsys total PSA reagent on Cobas e601 analyzers).

2.8 | RNA and protein extraction

Total RNA and protein were extracted using the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN), according to manufacturer's protocol and with protein fractions dissolved in 5% sodium dodecyl sulfate. RNA and protein concentrations were determined by absorbance measurements and RNA quality was verified with the 2100 Bioanalyzer (Agilent Technologies) as RNA integrity number ≥ 8 .

2.9 | Gene expression analysis

Gene expression profiles were obtained by human HT12 v4.0 Illumina Beadchip analysis, according to manufacturers' description (Illumina). Data analysis was performed with the GenomeStudio software (version 2011.1, Illumina). Samples were normalized using the average algorithm. Gene probes with average signals above two-time the mean background level in at least one sample were included, leaving 14 728 probes for further analysis. Differentially expressed genes were identified by the *t* test ($P < .05$). Genes defined to have a fold-change ≥ 2 were subjected to multivariate modeling and to functional pathway analysis.

2.10 | Multivariate modeling

Unsupervised principal component analysis (PCA) was used to create an overview of the variation in the transcription data and to detect clusters and trends among samples and expressed genes. Data were mean-centered before analysis, and models were validated by

sevenfold cross-validation. Multivariate statistical analysis was performed in SIMCA version 15.0.2 (Umetrics, Umeå, Sweden).

2.11 | Functional pathway analysis

Gene set enrichment analysis was performed by the MetaCore software (GeneGo, Thomson Reuters, New York, NY). Sets of genes associated with a functional process (pathway map) were determined as significantly enriched based on *P* values representing the probability for a process to arise by chance, considering the numbers of enriched gene products in the data versus the number of genes in the process. *P* values were adjusted by taking into account the rank of the process, given the total number of processes in the MetaCore ontology.

2.12 | Western blotting

Protein extracts from replicate samples were pooled in equal amounts and 10 to 50 µg protein per sample was separated by 4% to 20% Mini-PROTEAN TGX stain-free protein gels (Bio-Rad

Laboratories) and transferred onto a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories). Membranes were blocked in LI-COR blocking buffer (LI-COR Biosciences) before incubation with primary antibodies targeting ABCB1 (C219; BioLegend), AR (N-20; Santa Cruz Biotechnology), and β-actin (A5441, Sigma-Aldrich) followed by incubation with LI-COR Odyssey fluorescently labeled IRDye 800CW and IRDye 680RD secondary antibodies and analysis by LI-COR Odyssey CLx scanner and the ImageStudio version 3.1.4 software (LI-COR Biosciences).

2.13 | Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol followed by antigen retrieval using Tris-EDTA (pH 9) and blocking with Dako serum-free protein block (X0909; Dako) or Background Sniper (BS966L; Biocare Medical). Immunostaining was performed using primary antibodies targeting ATP-binding cassette sub-family B member 1

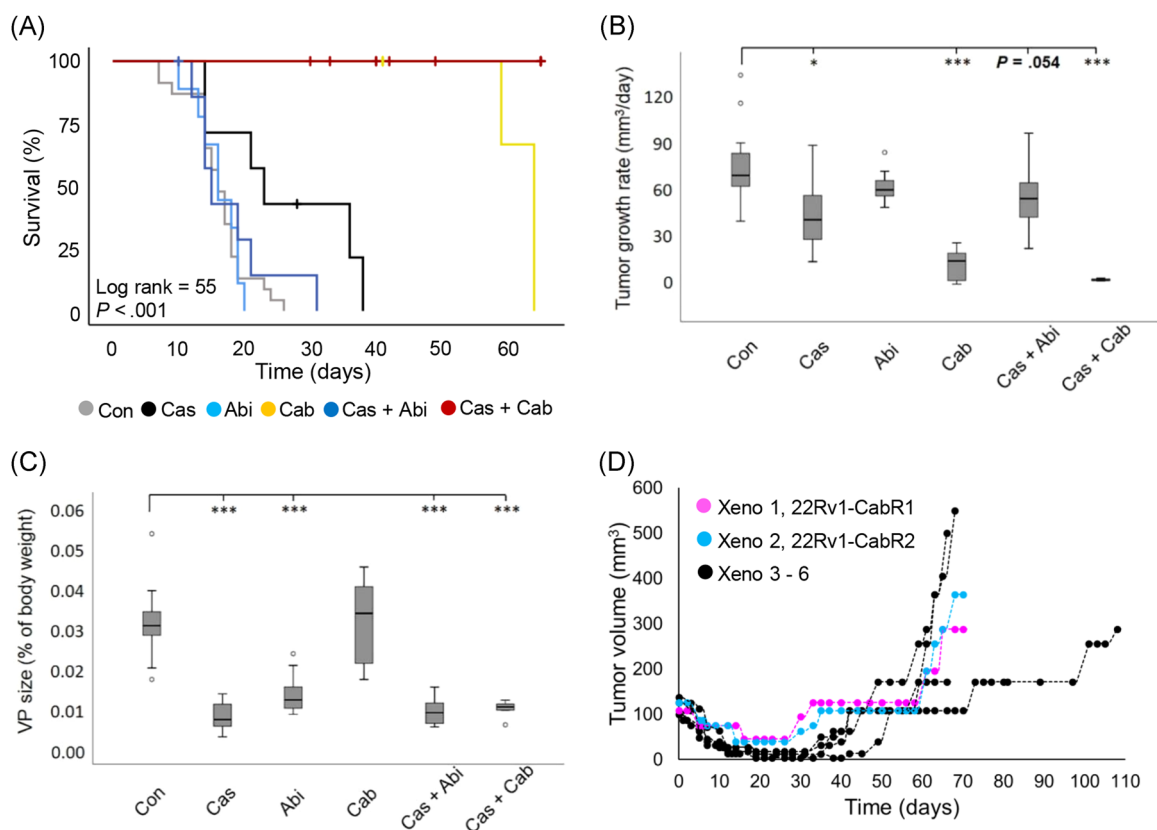


FIGURE 1 Tumor progression in 22Rv1 xenografts treated with vehicle and/or sham operation (Con, *n* = 23), surgical castration (Cas, *n* = 7), abiraterone (Abi, *n* = 9), cabazitaxel (Cab, *n* = 6), castration plus abiraterone (Cas + Abi, *n* = 8), or castration plus cabazitaxel (Cas + Cab, *n* = 11). A, Survival analysis of mice with 22Rv1 xenografts given different treatments for up to 65 days. Mice were killed when tumor volume reached approximately 1000 mm³. B, Tumor growth rate (mm³/day) until 65 days from treatment start or until tumor volume reached approximately 1000 mm³. C, Ventral prostate (VP) size (% of total body weight). D, Tumor volume (mm³) of individual xenografts (Xeno 1-6) repeatedly treated with cabazitaxel in castrated animals, initiated between Day 30 and 50 at tumor regrowth after the first round of cabazitaxel treatments (for details, see Section 2). Xenografts from which cell lines were established are specified. **P* < .05, ****P* < .001, in comparison to control group

(ABCB1) (C219; BioLegend), AR (N-20; Santa Cruz Biotechnology), or AR-V7 (31-1109-00; RevMab Biosciences) and the Envision horseradish peroxidase (HRP) Rabbit detection system (K4003; Dako) or the Rabbit-on-Rodent HRP-Polymer (RMR622H, Biocare Medical) with 3,3'-diaminobenzidine as chromogen and counterstaining with haematoxylin. Sections were scanned using the Panoramic 250 FLASH scanner and evaluated with the Panoramic viewer 1.15.2 software (3D HISTECH).

3 | RESULTS

3.1 | Cabazitaxel inhibits the growth of 22Rv1 xenografts

Effects of surgical castration, abiraterone acetate, cabazitaxel, and combinations thereof, were studied in 22Rv1 xenografts expressing high levels of constitutively active AR variants. After tumor establishment mice were treated with surgical castration ($n = 7$), abiraterone ($n = 9$), cabazitaxel ($n = 6$), castration plus abiraterone ($n = 8$), castration plus cabazitaxel ($n = 11$), or vehicle and/or sham operation ($n = 23$), and killed when tumor reached a volume of about 1000 mm³ (Figure S1A). Cabazitaxel treatment alone or in combination with castration significantly increased the survival of mice with 22Rv1 tumors (Figure 1A). Castration gave a modest survival benefit, while abiraterone had no obvious effect in this model (Figure 1A). This

was in line with markedly reduced tumor growth rates in mice treated with cabazitaxel (with or without castration) over a study time period up to 65 days (Figure 1B). Castration and castration in combination with abiraterone induced a modest reduction in growth rate, while abiraterone treatment did not significantly reduce the growth of 22Rv1 xenografts (Figure 1B), despite giving a castration effect comparable to that of surgical castration when monitored as reduced ventral prostate lobe weight (Figure 1C). Complete tumor regression after cabazitaxel treatment (+/- castration) was seen in three animals, while tumor regrowth was seen in 14 cases (>30 days). Six animals treated with castration plus cabazitaxel were subjected to repeated cabazitaxel treatment at tumor regrowth, and progress during cabazitaxel treatment was observed in all cases (Figure 1D).

3.2 | Establishment of cabazitaxel resistant cell lines

To study mechanisms behind cabazitaxel resistance, cells from three 22Rv1 xenograft tumors relapsing during cabazitaxel treatment were isolated and cultivated in vitro. Two stable cell lines were established; 22Rv1-CabR1 and 22Rv1-CabR2, which were subjected to further selection in vitro by gradually increasing cabazitaxel concentration up to 10 nmol/L in charcoal-stripped media. Resistance towards cabazitaxel was confirmed by a nine-day dose-response

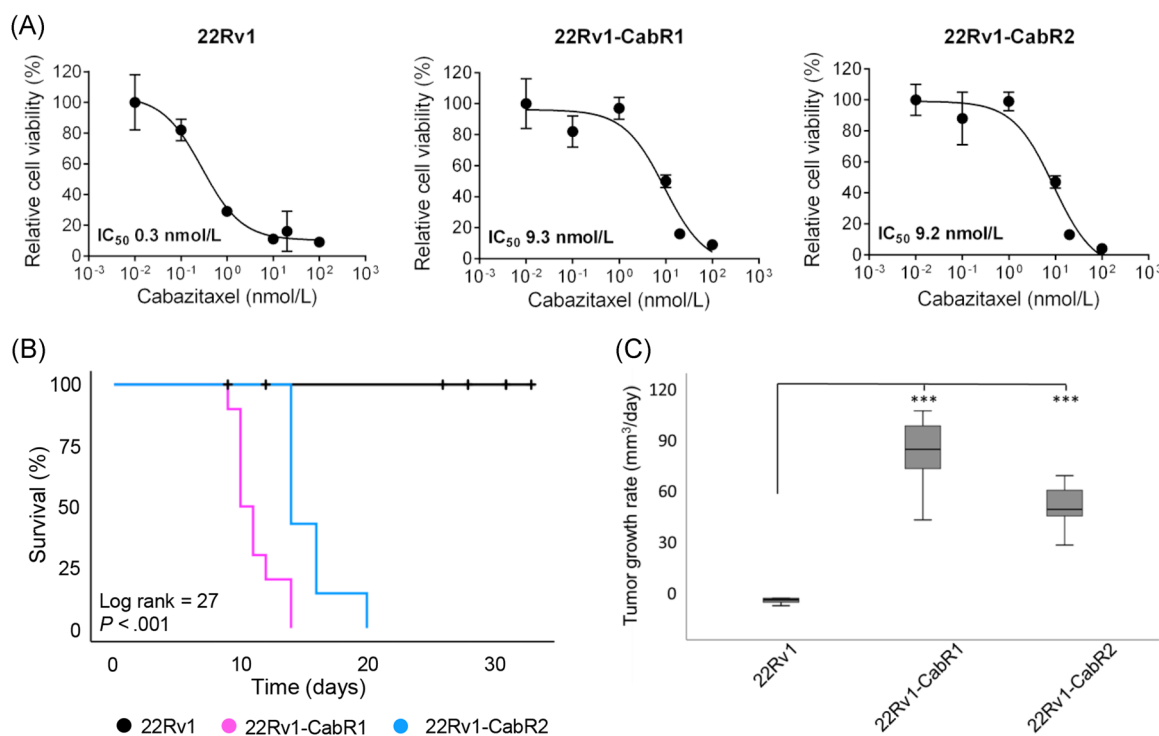


FIGURE 2 Cabazitaxel resistance is shown in two cell lines isolated from 22Rv1 xenografts relapsing during treatment with cabazitaxel. A, Dose-response curves for 22Rv1, 22Rv1-CabR1, and 22Rv1-CabR2 cells treated for 9 days with cabazitaxel in vitro. Experiments were performed in triplicates. Vertical bars indicate standard deviations. B, Survival analysis of mice with 22Rv1 ($n = 8$), 22Rv1-CabR1 ($n = 10$), and 22Rv1-CabR2 ($n = 7$) xenografts treated with cabazitaxel in castrated mice for up to 38 days. Mice were killed when tumor volume reached approximately 1000 mm³. C, Tumor growth rate (mm³/day) until 38 days from treatment start or until tumor volume approximately 1000 mm³. *** $P < .001$, in comparison to 22Rv1 xenografts. IC₅₀, half maximal inhibitory concentration [Color figure can be viewed at wileyonlinelibrary.com]

experiment showing half-maximal inhibitory concentration (IC_{50}) values of 9 nmol/L for 22Rv1-CabR1 and 22Rv1-CabR2 cells in comparison to 0.3 nmol/L for parental 22Rv1 cells (Figure 2A).

To investigate whether the cabazitaxel resistance displayed by 22Rv1-CabR1 and 22Rv1-CabR2 in vitro was sufficient to affect tumor growth in vivo, an additional round of xenograft experiments was performed. Mice were injected with 22Rv1-CabR1 ($n = 10$), 22Rv1-CabR2 ($n = 7$), or 22Rv1 cells used as control ($n = 8$). Before injection, cells were grown in charcoal-stripped media and mice were castrated. As seen in Figure 2B,C and Figure S1B, there was a clear difference in cabazitaxel

response/resistance manifested as rapid growth of the 22Rv1-CabR1 and 22Rv1-CabR2 xenografts during cabazitaxel treatment, while the control 22Rv1 xenografts clearly regressed.

3.3 | Mechanisms behind cabazitaxel resistance

To identify mechanisms behind cabazitaxel resistance, total RNA from 22Rv1-CabR1, 22Rv1-CabR2, and parental 22Rv1 cells cultured in charcoal-stripped media was analyzed in triplicates by

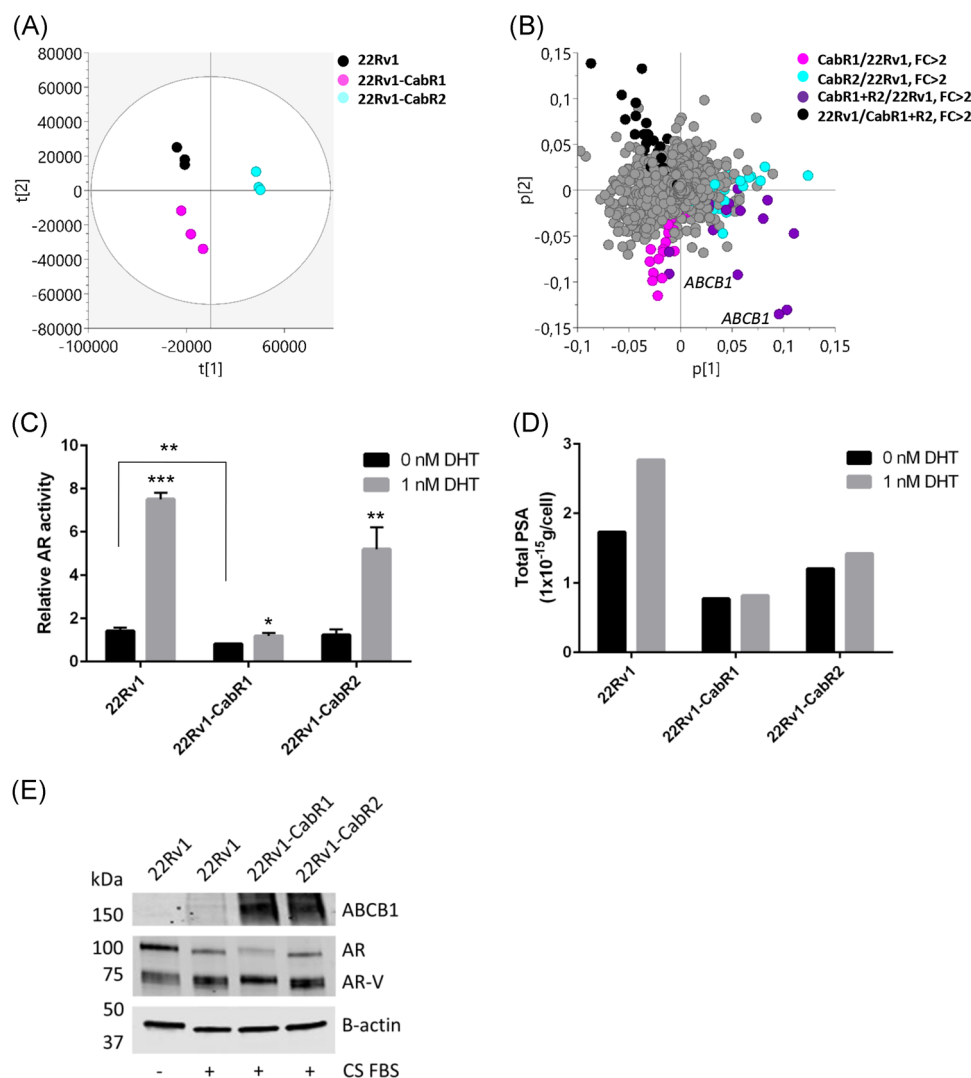


FIGURE 3 Gene expression patterns in 22Rv1-CabR1 and 22Rv1-CabR2 cell lines in comparison to 22Rv1 when grown in vitro. A, PCA score plot where each dot represents a cell line replicate, marked by different colors. B, Loading plot based on expression levels of 14 728 gene products responsible for the clustering of samples in A. Gene transcripts (dots) with a fold-change greater than 2 are marked by different colors, according to color code. Transcripts with positive loading values (p) are expressed at high levels in samples with positive score values (t) and vice versa. Transcripts for *ABCB1* (detected by two different gene probes) are indicated. C, AR activity was measured in the cell lines cultivated with or without DHT as indicated in the figure. To compensate for putative differences in transducing efficiency, the signals were related to signals from negative controls. * $P < .05$, ** $P < .01$, *** $P < .001$. D, PSA levels were measured in conditioned media from cells cultivated 4 days with or without 1 nM DHT. The total amount of PSA produced was then related to the number of cells per well. E, ABCB1, AR, and AR-V protein levels visualized by Western blot analysis of pooled protein fractions from triplicate cell line samples. β -actin was used as a loading control. ABCB1, ATP-binding cassette sub-family B member 1; AR, androgen receptor; CS FBS, charcoal-stripped FBS; DHT, dihydrotestosterone; FBS, fetal bovine serum; PCA, principal component analysis [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Genes with significantly increased expression levels ($P < .05$, $FC > 2$) in both cabazitaxel resistant cell lines; 22Rv1-CabR1 and 22Rv1-CabR2, in comparison to control 22Rv1 cell line

Gene probe symbol	Mean FC
ABCB1	86
ABCB1	57
TNFRSF19	13
NKX3-1	11
STEAP1	10
CNTNAP2	9.0
C7orf63	8.8
MGC87042	8.2
STEAP1	7.7
NMB	7.4
RUNDC3B	7.2
STEAP2	6.7
STEAP2	6.1
HTR3A	5.4
BASP1	5.1
SLC45A3	4.8
CSRP2	4.6
ILMN_1888359	4.3
ALDOC	4.2
HOXB8	4.2
EYA2	4.1
BAMBI	4.0
PRDM8	4.0
ANTXR2	3.9
LPL	3.6
BCHE	3.6
INSIG1	3.5
CKB	3.5
EVL	3.3
STC1	3.2
PDE9A	3.2
GABRA1	3.1
HMGC51	3.1
DACH1	3.1
CBLN2	3.0
C1orf116	3.0
ILMN_1825369	3.0
C1orf116	2.9
CDK6	2.8
HERC5	2.7
LOC653506	2.7
CORO2A	2.6
CHD5	2.5
NLGN1	2.5

(Continues)

TABLE 1 (Continued)

Gene probe symbol	Mean FC
IFITM3	2.5
GRIA2	2.5
ATP1B1	2.4
ID1	2.4
SLC25A37	2.3
ATP1B1	2.3
RAB27B	2.3
FLRT2	2.3
MAPT	2.3
PFKP	2.2
ACSS2	2.2
FAM189A1	2.2

Abbreviation: FC, fold-change.

whole-genome expression analysis. PCA analysis was used to identify common trends in expression patterns for the 22Rv1-CabR1 and 22Rv1-CabR2 cell lines in comparison to cabazitaxel responsive 22Rv1 cells. As can be seen in Figure 3A,B and Table 1, both 22Rv1-CabR1 and 22Rv1-CabR2 showed highly induced transcript levels of *ABCB1*. Western blot (Figure 3E) and immunohistochemical analysis (Figure 4) confirmed the upregulation of *ABCB1* protein expression in the cell membrane of 22Rv1-CabR1 and 22Rv1-CabR2.

The cabazitaxel resistant cell lines also showed induction of prostate associated transcripts (*NKX3-1*, *STEAP1*, *STEAP2*, and *SLC45A3*) (Table 1). As this might reflect a general increased in AR activity in 22Rv1-CabR1 and 22Rv1-CabR2 compared with parental 22Rv1 cells, the general AR activity in the cell lines were examined using an AR reporter assay in the absence and presence of DHT. Surprisingly, the resistant cell lines showed lower endogenous AR activity than the parental 22Rv1 cells and also less induction by DHT stimulation (Figure 3C). A similar tendency was seen for PSA secretion; with 22Rv1 producing the highest and 22Rv1-CabR1 the lowest PSA amount per cell (Figure 3D). In line with this, the 22Rv1-CabR1 cell line showed lower AR levels than the other cell lines (Figure 3E and Figure 4). Notably, the AR-V levels did not obviously differ in relation to cabazitaxel resistance.

Pathway analysis of differentially expressed genes in the cabazitaxel resistant cell lines (Table S1) indicated a strong upregulation of the SCAP/SREBP transcriptional control of cholesterol and fatty acid biosynthesis in 22Rv1-CabR2, but not in 22Rv1-CabR1 cells (Table S2).

3.4 | Anti-androgens partly restores susceptibility to cabazitaxel

To confirm that the cabazitaxel resistance in 22Rv1-CabR1 and 22Rv1-CabR2 was caused by increased *ABCB1* expression, cells were

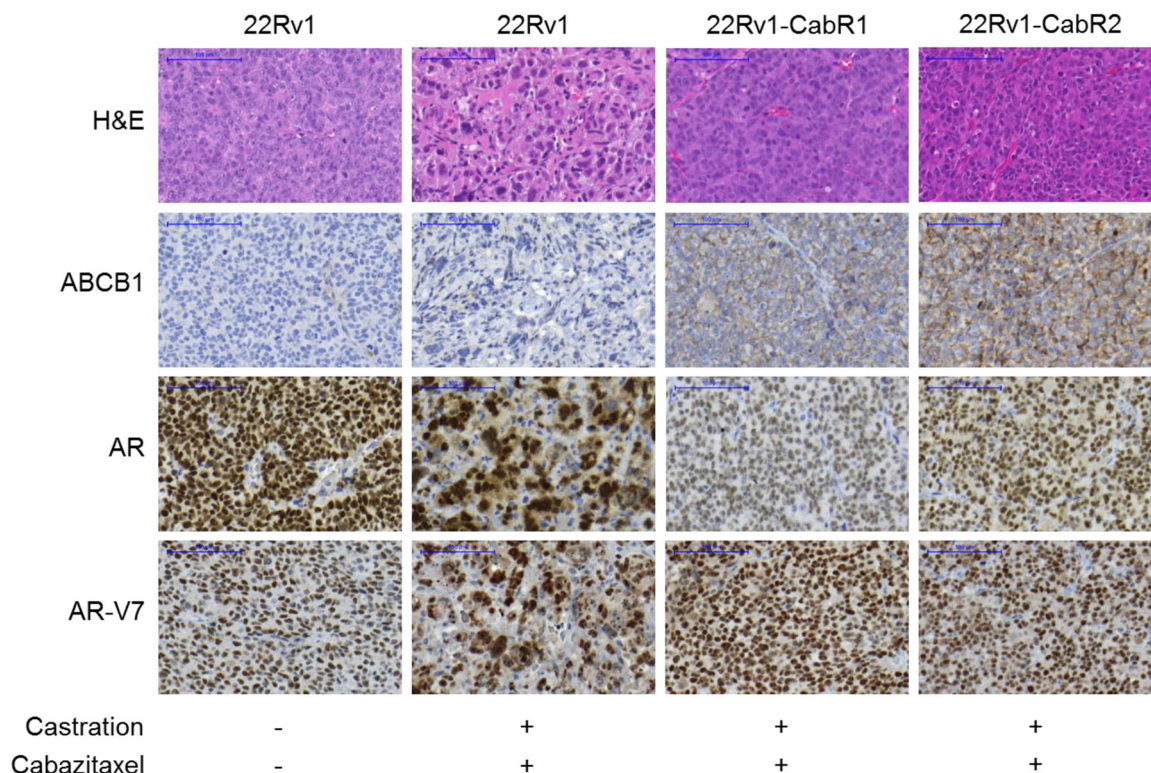


FIGURE 4 Immunohistochemical staining showing ABCB1, AR, and AR-V7 protein expression in 22Rv1, 22Rv1-CabR1, and 22Rv1-CabR2 xenograft tumors, with castration and cabazitaxel treatment as indicated (-/+). Bar indicates 100 μ m. ABCB1, ATP-binding cassette sub-family B member 1; AR, androgen receptor; H&E, hematoxylin and eosin staining

incubated with the ABCB1 inhibitor elacridar (0.25 μ mol/L) during cabazitaxel treatment up to 10 nmol/L for 96 hours. Elacridar restored the cabazitaxel susceptibility of 22Rv1-CabR1 and 22Rv1-CabR2 cells to that of the parental 22Rv1 cell line (Figure 5A-C). Interestingly, AR-antagonists have been shown to reverse ABCB1-mediated taxane resistance^{20,21} and this was also the case here. Both bicalutamide (20 μ mol/L) and enzalutamide (20 μ mol/L) significantly increased the response of 22Rv1-CabR1 and 22Rv1-CabR2 cells to cabazitaxel although not as efficient as elacridar (Figure 5). Both bicalutamide and enzalutamide were slightly more effective in restoring cabazitaxel sensitivity in 22Rv1-CabR2 (Figures 5F,I) than in 22Rv1-CabR1 cells (Figures 5E,H). In contrast, neither AR-antagonists nor elacridar affected the cabazitaxel sensitivity in 22Rv1 control cells (Figures 5A, 5D, and 5G).

3.5 | Simvastatin resistance in cabazitaxel resistant cells

In light of the pathway analysis showing upregulation of cholesterol and fatty acid biosynthesis in 22Rv1-CabR2 cells, cells were treated with simvastatin; a lipid-lowering drug acting by inhibiting HMG-CoA reductase and with potential beneficial effects on patients with prostate cancer.²² Accordingly, 22Rv1-CabR2 cells were more resistant to simvastatin treatment (IC_{50} of 27 nmol/L) than 22Rv1-CabR1 (IC_{50} = 8.9 nmol/L) and 22Rv1 (IC_{50} = 5.9 nmol/L) cells (Figure 6).

4 | DISCUSSION

In this study, we used the 22Rv1 xenograft model to test the efficiency of abiraterone and cabazitaxel treatment on prostate tumors expressing high levels of the constitutively active ligand-binding domain (LBD)-truncated AR variants, such as AR-V7. As anticipated abiraterone had no significant effect on 22Rv1 tumor growth as it acts by inhibiting androgen synthesis and thus indirectly has the ability to inhibit ligand binding to the full-length AR, but not to its LBD-truncated variants. Our results are in line with previous studies showing that tumor expression of constitutive active AR variants is associated with resistance to abiraterone treatment.^{10-13,23} Cabazitaxel, on the other hand, inhibited 22Rv1 tumor growth by itself and in combination with castration, which is also in line with clinical observations where patients with CRPC show response to cabazitaxel independent of AR-V7 tumor status.^{12,14,15} Surgical castration moderately reduced the growth of 22Rv1 xenografts, probably by reducing testosterone levels and inhibiting activation of the wild type AR. Why abiraterone was not as effective as surgical castration in inhibiting xenograft growth, despite a castrate effect in the ventral prostate lobe, is not known.

Despite the initial good response to cabazitaxel, tumor regrowth was eventually seen in the majority of 22Rv1 xenografts treated. As tumors continued to grow even in animals receiving repeated cabazitaxel treatment, cabazitaxel resistance was suspected and,

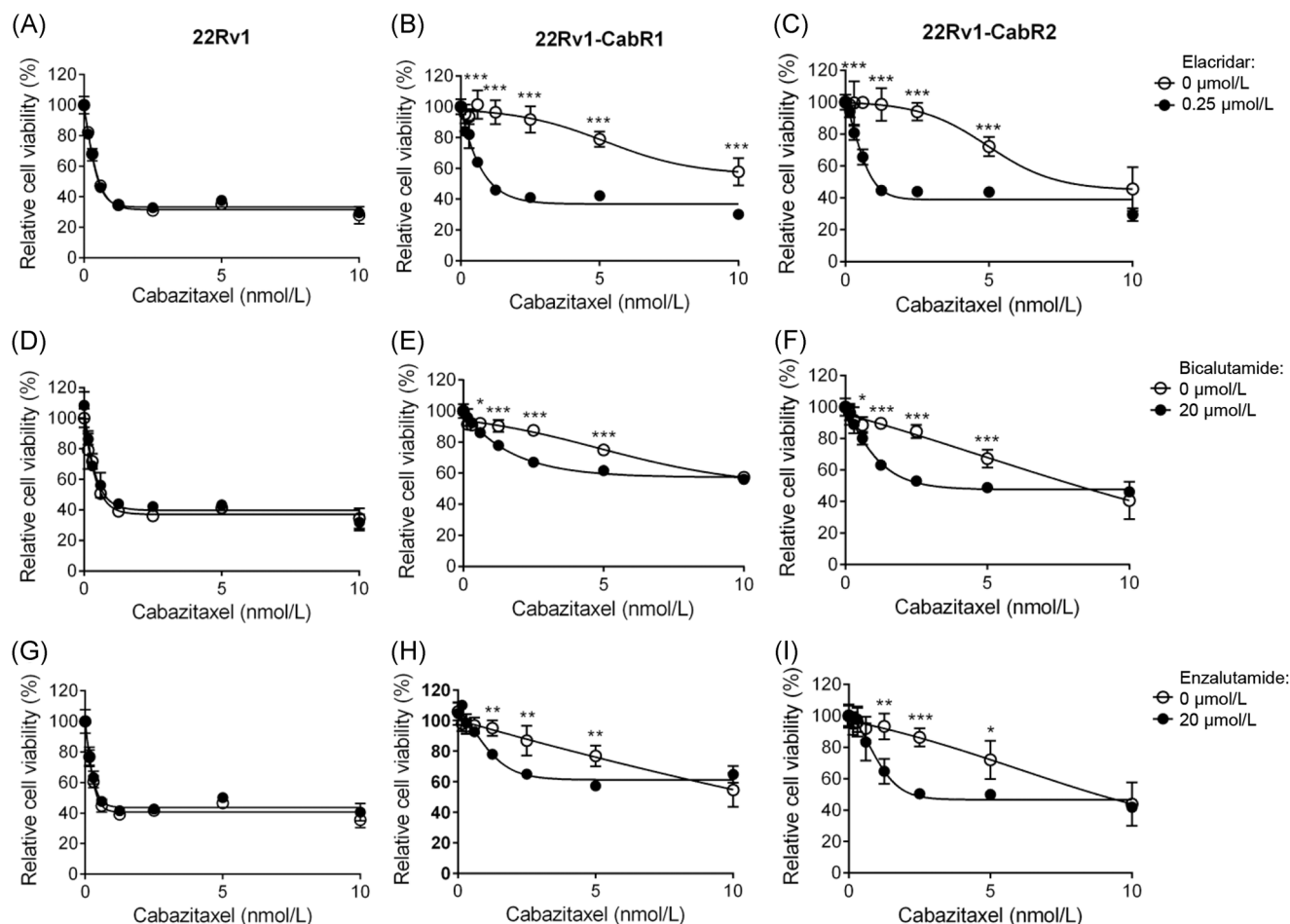


FIGURE 5 22Rv1, 22Rv1-CabR1, and 22Rv1-CabR2 cells treated with ABCB1 inhibitor elacridar (A–C) or AR-antagonists; bicalutamide (D–F) and enzalutamide (G–I) for 96 hours in combination with up to 10 nmol/L cabazitaxel. Experiments were performed in quadruplicates. Vertical bars indicate standard deviations. * $P < .05$, ** $P < .01$, *** $P < .001$, in comparison to vehicle. ABCB1, ATP-binding cassette sub-family B member 1; AR, androgen receptor

subsequently, proved both in vitro and in vivo for two established cell lines; 22Rv1-CabR1 and 22Rv1-CabR2.

Analysis of gene expression data from the cabazitaxel resistant cell lines showed clearly induced levels of *ABCB1* mRNA. The *ABCB1* gene codes for the ABCB1 protein that is also known as the multidrug resistance protein 1 or the P-glycoprotein with known

function as a drug efflux pump.²⁴ The ABCB1 protein has been shown to mediate acquired docetaxel-resistance in several prostate cancer models.^{20,25,26} Cabazitaxel shows less ABCB1 affinity than docetaxel and was initially chosen for clinical development based on its activity in the taxane-resistant model system.^{27,28} Still, ABCB1 has been shown to mediate cross-resistance to cabazitaxel in docetaxel-

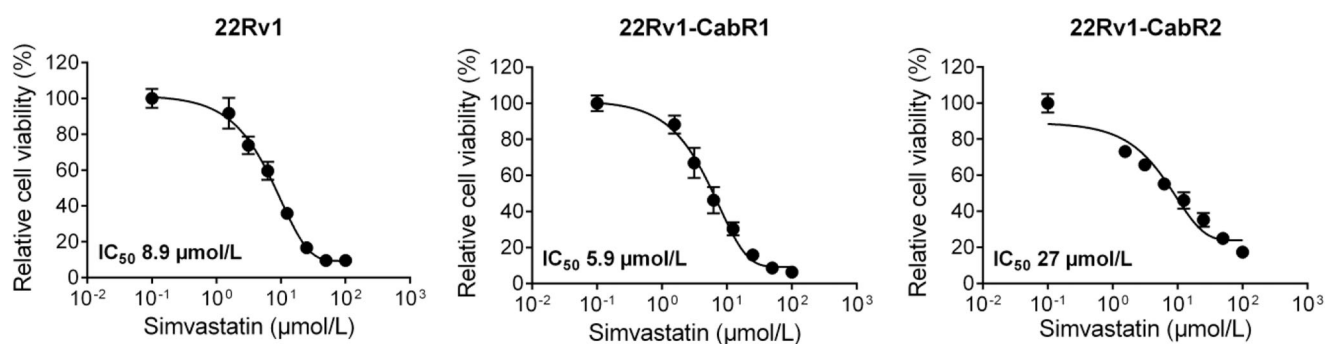


FIGURE 6 Dose-response curves of 22Rv1, 22Rv1-CabR1, and 22Rv1-CabR2 cells to 96 hours of simvastatin treatment. Experiments were performed in quadruplicates. Vertical bars indicate standard deviations. IC₅₀, half-maximal inhibitory concentration

resistant LNCaP-C42B and DU145 cells²¹ and acquired resistance have been reported as well.²⁹ Here we contribute with novel findings showing that induced expression of ABCB1 is associated with acquired resistance to cabazitaxel in the 22Rv1 xenograft model. Furthermore, we verify recent findings showing that anti-androgens, bicalutamide, and enzalutamide, can be used to resensitize prostate cancer cells to taxane treatment.^{20,21} Those findings may have great clinical potential, and raise the question if anti-androgens should be given in sequence with taxanes to prolong time to resistance. Another possibility would be to give anti-androgens in adjuvant settings to reduce the efficient taxane dose in patients. The toxicity of taxanes, in general, restrict their usage in patients with comorbidity and even a modest reduction of cabazitaxel dosage may be clinically relevant in patients with comorbidity who otherwise would be denied life-prolonging chemotherapy.

Bicalutamide and enzalutamide have been shown to inhibit ABCB1 efflux activity independent of AR status,²⁰ but it might be worth noting that full-length AR expression was higher in 22Rv1-CabR2 than in 22Rv1-CabR1, that is, in the cell line more susceptible to the reversion of cabazitaxel resistance through anti-androgens. Levels of AR variants did not change from castration, abiraterone or cabazitaxel treatment and neither were levels changed in the cabazitaxel resistant cell lines, indicating that AR variants, including AR-V7, are not involved in the development of taxane resistance. This is in line with a recent study showing that overexpression of AR-V7 in C42B cells did not mediate taxane resistance.³⁰

Notably, the cabazitaxel resistant cell lines 22Rv1-CabR2 and 22Rv1-CabR1 demonstrated less AR activity compared with the parental 22Rv1 cell line, measured both as general AR activity and as PSA production or secretion. In contrast, the resistant cell lines contained higher transcript levels of some genes known to be positively regulated by androgens, for example, *NKX3-1*, *STEAP1*, *STEAP2*, and *SLC45A3*. If and how these findings are relevant for acquired cabazitaxel resistance remains to be explored. One possibility is that they reflect cellular differentiation state and divergent regulation of AR regulated genes.

Besides induced expression of efflux transporters, other reported mechanisms for taxane resistance include accumulation of β -tubulins, alterations of survival factors and apoptosis regulators, downregulation of BRCA1, and epithelial-to-mesenchymal transition.³¹ Neither of these pathways appeared markedly affected in 22Rv1-CabR1 or 22Rv1-CabR2 cells. Instead, functional pathway analysis clearly indicated the upregulation of cholesterol and fatty acid biosynthesis in 22Rv1-CabR2 cells. Accordingly, treatment using the cholesterol-lowering drug simvastatin showed that 22Rv1-CabR2 cells were three to five times less sensitive than 22Rv1-CabR1 and 22Rv1 cells, respectively, evidentially due to higher levels of molecules within the targeted pathway. Those findings are in line with recent observations from our group demonstrating high cholesterol levels and β -oxidation in clinical samples of CRPC metastases.³²⁻³⁴

In conclusion, this study shows initially pronounced response to cabazitaxel in the prostate cancer 22Rv1 xenograft model expressing constitutively active, LBD-truncated AR variants (including AR-V7).

Sub-sequential development of cabazitaxel resistance was associated with induced expression of the ABCB1 drug efflux transporter as well as by increased cholesterol biosynthesis. Resistance could be reversed by the ABCB1 inhibitor elacridar and partly overcome by coadministration of the AR-antagonists bicalutamide and enzalutamide. Taken together, our results show great translational potential suggesting that combined treatment with taxanes and anti-androgens could be used to overcome and/or delay acquired cabazitaxel resistance in patients with prostate cancer.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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